TRANSLATOR'S DECLARATION

I, Janet Hope, BSc(Hons.), MIL., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 44 pages of a German Patent application in the German language with the title:

Neue für das metY-Gen kodierende Nukleotidsequenzen

identified by the code number 000053 BT at the upper left of each page and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

Signed:

Dated: 2nd March 2004

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The attached papers are a true and accurate reproduction of the original documents for this patent application.

Munich, 12th June 2001

On behalf of the President of the German Patent and Trade Mark Office

(signature)

Weihmayr

New nucleotide sequences which code for the metY gene

The invention provides nucleotide sequences from coryneform bacteria which code for the metY gene and a process for the fermentative preparation of amino acids, in particular L-lysine and L-methionine, using bacteria in which at least the metY gene is enhanced.

Prior art

L-Amino acids, in particular L-lysine and L-methionine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and produce amino acids are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains which produce L-amino acid, by

amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids, in particular L-lysine and L-methionine.

Description of the invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine.

When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

When L-methionine or methionine are mentioned in the following, the salts, such as e.g. methionine hydrochloride or methionine sulfate, are also meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the metY gene, chosen from the group consisting of

a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to

the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of O-acetylhomoserine sulfhydrylase.

The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

The invention also provides

a polynucleotide comprising the nucleotide sequence as shown in SEQ ID No. 1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the DNA sequence of C.glutamicum which codes for the metY gene, deposited in accordance with the Budapest Treaty in Corynebacterium glutamicum as pCREmetY on 13.05.00 under DSM 13556

and coryneform bacteria in which the metY gene is present in enhanced form, in particular by the vector pCREmetY.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for O-acetylhomoserine sulfhydrylase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence with that of the O-acetylhomoserine sulfhydrylase gene.

Polynucleotide sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for O-acetylhomoserine sulfhydrylase can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of O-acetylhomoserine sulfhydrylase, and also those which are at least 70%, preferably at least 80%, and in particular which are at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention moreover provides a process for the fermentative preparation of amino acids, in particular L-lysine and L-methionine, using coryneform bacteria which in particular already produce amino acids, and in which at least the nucleotide sequences which code for the metY gene are enhanced, in particular over-expressed.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-amino acids, in particular L-lysine and L-methionine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus Corynebacterium. Of the genus Corynebacterium, there may be mentioned in particular the species Corynebacterium glutamicum, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum (C. glutamicum), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

or L-lysine-producing mutants or strains prepared therefrom, such as, for example

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464 and
Corynebacterium glutamicum DSM5715.

or L-methionine-producing mutants or strains prepared therefrom, such as, for example

Corynebacterium glutamicum ATCC21608.

The inventors have succeeded in isolating the new metY gene from C. glutamicum which codes for the enzyme O-acetylhomoserine sulfhydrylase (EC 4.2.99.10).

To isolate the metY gene or also other genes of C. glutamicum, a gene library of this microorganism is first set up in Escherichia coli (E. coli). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the E. coli K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of C. glutamicum ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326)) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)). To prepare a gene library of C. glutamicum in E. coli it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those E. coli strains which are restriction- and recombination-defective. An example of these is the strain DH5αmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then

sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of C. glutamicum which codes for the metY gene and which, as SEQ ID No. 1, is a constituent of the present invention has been obtained in this manner. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the metY gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a

corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991), 41: 255-260). The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approx. 50 - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim,

Mannheim, Germany, 1995) a temperature of approx. 50 - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise from 50 to 68°C in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids, in particular L-lysine and L-methionine, in an improved manner after over-expression of the metY gene, optionally in combination with the metA gene.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-lysine and L-methionine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying

number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, for enhancement the metY gene according to the invention was over-expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891), can be used in the same manner.

Examples of such plasmid vectors are shown in figures 1 and 2.

Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al.,1986, Gene 41: 337-342). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

In addition, it may be advantageous for the production of amino acids, in particular L-lysine and L-methionine, to enhance one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, or of amino acid export, in addition to the metY gene.

Thus, for example, for the preparation of L-lysine, one or more genes chosen from the group consisting of

Thus, for example, for the preparation of L-lysine one or more genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- the lysC gene which codes for a feed-back resistant aspartate kinase (ACCESSION NUMBER P26512),

can be enhanced, in particular over-expressed.

Thus, for example, for the preparation of L-methionine one or more genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),

- the lysC gene which codes for a feed-back resistant aspartate kinase (ACCESSION NUMBER P26512),
- the metA gene which codes for homoserine O-acetyltransferase (ACCESSION Number AF052652),
- the metB gene which codes for cystathionine gamma-synthase (ACCESSION Number AF126953),
- the aecD gene which codes for cystathionine gamma-lyase (ACCESSION Number M89931)
- the glyA gene which codes for serine hydroxymethyltransferase (JP-A-08107788),

can be enhanced, in particular over-expressed, additional enhancement of metA being particularly preferred.

It may furthermore be advantageous for the production of L-lysine, in addition to the enhancement of the metY gene, for one or more genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114)

to be attenuated, in particular for the expression thereof to be reduced.

It may furthermore be advantageous for the production of L-methionine, in addition to the enhancement of the metY gene, for one or more genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),

- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114)
- the thrB gene which codes for homoserine kinase (ACCESSION Number P08210),
- the ilvA gene which codes for threonine dehydratase (ACCESSION Number Q04513),
- the thrC gene which codes for threonine synthase (ACCESSION Number P23669),
- the ddh gene which codes for meso-diaminopimelate D-dehydrogenase (ACCESSION Number Y00151),

to be attenuated, in particular for the expression thereof to be reduced.

In addition to over-expression of the metY gene, optionally in combination with the metA gene it may furthermore be advantageous, for the production of amino acids, in particular L-lysine and L-methionine, to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids, in particular L-lysine and L-methionine. A summary of known culture methods are described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart,

1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/ Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substance can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to

the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-lysine and L-methionine can be carried out by ion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190).

The following microorganism was deposited as a pure culture on 13.05.00 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Corynebacterium glutamicum strain DSM5715/pCREmetY as DSM 13556

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-lysine and L-methionine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

Example 1

Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences, USA, 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al.

(1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2

Isolation and sequencing of the metY gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol. Letters, 123:343-7) into the E. coli strain DH5αmcr (Grant, 1990, Proceedings of the National Academy of

Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1313 base pairs, which was called the metY gene. The metY gene codes for a protein of 437 amino acids.

Example 3

Construction of vectors for expression of metY and metAY

3.1. Amplification of the genes metY and metA

The methionine biosynthesis genes metA and metY from C. glutamicum were amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequences of the methionine biosynthesis genes metY (SEQ ID No.1) and metA (gene library entry Accession Number AF052652) of C. glutamicum ATCC 13032, PCR primers were synthesized (MWG Biotech, Ebersberg, Germany). These primers were chosen so that the amplified fragments contain the genes and native ribosome binding sites thereof, but not possible promoter regions. In addition, suitable restriction cutting sites which allow cloning into the target vector were inserted. The sequences of the PCR primers, the cleavage sites inserted (sequence underlined) and the amplified gene (the fragment size, in bp, is listed in parentheses) are listed in the following table 1.

Table 1

Primer	Sequence with restriction cleavage site	Product	Plasmid
metY-EVP5	5'-CTAATAAGTCGACAAAGGAGGACA SalI ACCATGCCAAAGTACGAC- 3'	metY (1341 bp)	pCREmetY
metY-EVP3	5'-GAGTCTAATGCATGCTAGATTGCA NsiI GCAAAGCCG 3'		
metA-EVP5	5'-AGAACGAATTCAAAGGAGGACAAC EcoRI CATGCCCACCCTCGCGC-3'	metA (1161 bp)	pCREmetA
metA-EVP3	5'-GTCGTGGATCCCCTATTAGATGTA PstI GAACTCG-3'		

The PCR experiments were carried out with the Taq DNA polymerase from Gibco-BRL (Eggestein, Germany) in a "PCT-100 Thermocycler" (MJ Research Inc., Watertown, Mass., USA). A single denaturing step of 2 minutes at 94°C was followed by a denaturing step of 90 seconds (sec) at 94°C, an annealing step for 90 sec at a primer-dependent temperature of T=(2xAT+4xGC) -5 C (Suggs, et al., 1981, p. 683-693, In: D.D. Brown, and C.F. Fox (Eds.), Developmental Biology using Purified Genes. Academic Press, New York, USA) and an extension step at 72°C lasting 90 sec. The last three steps were repeated as a cycle 35 times and the reaction was ended with a final extension step of 10 minutes (min) at 72°C. The products amplified in this way were tested electrophoretically in a 0.8% agarose gel.

The metY fragment 1341 bp in size was cleaved with the restriction endonucleases SalI and NsiI, and the metA fragment 1161 bp in size was cleaved with the restriction endonucleases EcoRI and BamHI. The two batches were separated by gel electrophoresis and the fragments metY

(approx. 1330 bp) and metA (approx. 1150 bp) were isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

3.2. Cloning of metY in the vector pZ8-1

The E. coli - C. glutamicum shuttle expression vector pZ8-1 (EP 0 375 889) was employed as the base vector for expression both in C. glutamicum and in E. coli. DNA of this plasmid was cleaved completely with the restriction enzymes SalI and PstI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). The metY fragment isolated from the agarose gel in example 3.1 was mixed with the vector pZ8-1 prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

The ligation batch was transformed in the E. coli strain DH5α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetY. It is shown in figure 1.

3.3. Cloning of metA and metY in the vector pZ8-1

DNA of the plasmid pZ8-1 was cleaved completely with the restriction enzymes EcoRI and BamHI and then dephosphorylated with shrimp alkaline phosphatase (Roche

Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). The metA fragment isolated from the agarose gel in example 3.1 was mixed with the vector pZ8-1 prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

The ligation batch was transformed in the E. coli strain DH5α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetA.

The plasmid pCREmetA was cleaved completely with the restriction enzymes SalI and PstI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). The metY fragment isolated from the agarose gel in example 3.1 was mixed with the vector pCREmetA prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

The ligation batch was transformed in the E. coli strain DH5α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the

Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetAY. It is shown in figure 2.

Example 4

Preparation of the strains DSM5715/pCREmetY and DSM5715/pCREmetAY

The vectors pCREmetY and pCREmetAY mentioned in example 3.2 and 3.3 were electroporated by the electroporation method of Tauch et al. (1994, FEMS Microbiological Letters, 123:343-347) in Corynebacterium glutamicum DSM 5715. The strain DSM 5715 is an AEC-resistant lysine producer. Selection for plasmid-carrying cells was made by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated in each case from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927) and checked by restriction cleavage with subsequent agarose gel electrophoresis. The strains were called DSM5715/pCREmetY and DSM5715pCREmetAY. The strain DSM5715/pCREmetY has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 13556.

Example 5

Preparation of lysine with the strain DSM5715/pCREmetY

The C. glutamicum strain DSM5715/pCREmetY obtained in example 4 was cultured in a nutrient medium suitable for

the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (50 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Kanamycin (50 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660 nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4$ * 7 H_2O	1.0 g/l
$CaCl_2$ * 2 H_2O	10 mg/l
$FeSO_4$ * 7 H_2O	10 mg/l
$MnSO_4$ * H_2O	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (50 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-

BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in table 2.

Table 2

Strain	OD(660)	Lysine HCl g/l
DSM5715	10.6	15.7
DSM5715/pCREmetY	9.5	16.1

<u>Example 6</u>

Preparation of methionine with the strain DSM5715/pCREmetAY

The C. glutamicum strain DSM5715/pCREmetAY obtained in example 4 was cultured in a nutrient medium suitable for the production of methionine and the methionine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (50 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII as described in example 5 was used as the medium for the preculture.

Kanamycin (50 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660 nm) of the main culture was 0.1. The medium MM as described in example 5 was used for the main culture.

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (50 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of methionine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in table 3.

Table 3

Strain	OD(660)	Methionine g/l
DSM5715	6.6	1.4
DSM5715/pCREmetAY	8.3	16.0

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gtctattgca atagaccaag ctgttcagta gggtgcatgg gagaagaatt tcctaataaa 180

aactcttaag gacctccaa atg cca aag tac gac aat tcc aat gct gac cag  232
                     Met Pro Lys Tyr Asp Asn Ser Asn Ala Asp Gln
                       1               5                  10

tgg ggc ttt gaa acc cgc tcc att cac gca ggc cag tca gta gac gca   280
Trp Gly Phe Glu Thr Arg Ser Ile His Ala Gly Gln Ser Val Asp Ala
            15                  20                  25

cag acc agc gca cga aac ctt ccg atc tac caa tcc acc gct ttc gtg   328
Gln Thr Ser Ala Arg Asn Leu Pro Ile Tyr Gln Ser Thr Ala Phe Val
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Phe Asp Ser Ala Glu His Ala Lys Gln Arg Phe Ala Leu Glu Asp Leu
    45                  50                  55

ggc cct gtt tac tcc cgc ctc acc aac cca acc gtt gag gct ttg gaa   424
Gly Pro Val Tyr Ser Arg Leu Thr Asn Pro Thr Val Glu Ala Leu Glu
 60                  65                  70                  75

aac cgc atc gct tcc ctc gaa ggt ggc gtc cac gct gta gcg ttc tcc   472
Asn Arg Ile Ala Ser Leu Glu Gly Gly Val His Ala Val Ala Phe Ser
                 80                  85                  90
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Ser Gly Gln Ala Ala Thr Thr Asn Ala Ile Leu Asn Leu Ala Gly Ala
95 100 105

ggc gac cac atc gtc acc tcc cca cgc ctc tac ggt ggc acc gag act 568
Gly Asp His Ile Val Thr Ser Pro Arg Leu Tyr Gly Gly Thr Glu Thr
110 115 120

cta ttc ctt atc act ctt aac cgc ctg ggt atc gat gtt tcc ttc gtg 616
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Glu Asn Pro Asp Asp Pro Glu Ser Trp Gln Ala Ala Val Gln Pro Asn
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Thr Lys Ala Phe Phe Gly Glu Thr Phe Ala Asn Pro Gln Ala Asp Val
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Asn Ile Gly Asp Val Arg Ser Leu Val Val His Pro Ala Thr Thr Thr
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tcc acc gtc cgc ctg tcc gtt ggc atc gag acc att gat gat atc atc 1480
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Tyr His Gly Leu Lys Tyr Ala Asp Leu Gly Ala Pro Ala Phe Gly Leu
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Lys Val Arg Val Gly Leu Leu Arg Asp Thr Gly Ser Thr Leu Ser Ala
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Phe Asn Ala Trp Ala Ala Val Gln Gly Ile Asp Thr Leu Ser Leu Arg
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Leu Glu Arg His Asn Glu Asn Ala Ile Lys Val Ala Glu Phe Leu Asn
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Asn His Glu Lys Val Glu Lys Val Asn Phe Ala Gly Leu Lys Asp Ser
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Pro Trp Tyr Ala Thr Lys Glu Lys Leu Gly Leu Lys Tyr Thr Gly Ser
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Val Leu Thr Phe Glu Ile Lys Gly Gly Lys Asp Glu Ala Trp Ala Phe
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Ile Asp Ala Leu Lys Leu His Ser Asn Leu Ala Asn Ile Gly Asp Val
370 375 380

Arg Ser Leu Val Val His Pro Ala Thr Thr Thr His Ser Gln Ser Asp
385 390 395 400

Glu Ala Gly Leu Ala Arg Ala Gly Val Thr Gln Ser Thr Val Arg Leu
405 410 415

Ser Val Gly Ile Glu Thr Ile Asp Asp Ile Ile Ala Asp Leu Glu Gly
420 425 430

Gly Phe Ala Ala Ile
435

The following figures are attached:

- Figure 1: Plasmid pCREmetY
- Figure 2: Plasmid pCREmetAY

The abbreviations used in the figures have the following meaning:

Kan:	Resistance gene for kanamycin
metY:	metY gene of C. glutamicum
metA:	metA gene of C. glutamicum
Ptac:	tac promoter
rrnB-T1T2:	Terminator T1T2 of the rrnB gene of E.coli
rep:	Plasmid-coded replication origin for C. glutamicum (of pHM1519)
BamHI:	Cleavage site of the restriction enzyme BamHI
EcoRI:	Cleavage site of the restriction enzyme EcoRI
EcoRV:	Cleavage site of the restriction enzyme EcoRV
PstI:	Cleavage site of the restriction enzyme PstI
SalI:	Cleavage site of the restriction enzyme SalI
XhoI:	Cleavage site of the restriction enzyme XhoI

Patent claims

1. An isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the metY gene, chosen from the group consisting of

   a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

   b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

   c) polynucleotide which is complementary to the polynucleotides of a) or b), and

   d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

   the polypeptide preferably having the activity of O-acetylhomoserine sulfhydrylase.

2. The polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.

3. The polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.

4. The polynucleotide as claimed in claim 2 or 3, comprising the nucleic acid sequence as shown in SEQ ID No. 1.

5. The DNA as claimed in claim 2 or 3 which is capable of replication, comprising

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

6. The DNA as claimed in claim 5 which is capable of replication, wherein the hybridization of sequence (iii) is carried out under a stringency corresponding to at most 2x SSC.

7. The polynucleotide sequence as claimed in claim 2 or 3, which codes for a polypeptide which comprises the amino acid sequence in SEQ ID No. 2.

8. A process for the fermentative preparation of L-amino acids, in particular L-lysine, wherein the following steps are carried out:

a) fermentation of the coryneform bacteria which produce the desired amino acid and in which at least the metY gene or nucleotide sequences which code for it are enhanced, in particular over-expressed;

b) concentration of the L-amino acid in the medium or in the cells of the bacteria; and

c) isolation of the L-amino acid.

9. A process for the fermentative preparation of L-amino acids, in particular L-methionine, wherein the following steps are carried out:

a) fermentation of the L-methionine-producing coryneform bacteria in which the metY gene, optionally with met A, is enhanced, in particular over-expressed;

b) concentration of the L-amino acid in the medium or in the cells of the bacteria; and

c) isolation of the L-amino acid.

10. The process as claimed in claim 8 or 9, wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.

11. The process as claimed in claim 8 or 9, wherein bacteria in which the metabolic pathways which reduce the formation of the desired amino acid are at least partly eliminated are employed.

12. The process as claimed in claim 8, wherein a strain transformed with a plasmid vector is employed, and the plasmid vector carries the metY gene and optionally additionally the metA gene.

13. The process as claimed in claim 9, wherein a strain transformed with a plasmid vector is employed, and the plasmid vector carries the nucleotide sequence which codes for the metA and metY genes.

14. The process as claimed in claim 8, wherein for the preparation of L-amino acids, in particular L-lysine, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of

14.1 the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,

14.2 the tpi gene which codes for triose phosphate isomerase,

14.3 the pgk gene which codes for 3-phosphoglycerate kinase

14.4 the pyc gene which codes for pyruvate carboxylase

14.5 the lysC gene which codes for a feed back resistant aspartate kinase,

is or are enhanced, in particular over-expressed, are fermented.

15. The process as claimed in claim 9, wherein for the preparation of L-amino acids, in particular L-methionine, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of

15.1 the lysC gene which codes for a feed back resistant aspartate kinase,

15.2 the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,

15.3 the tpi gene which codes for triose phosphate isomerase,

15.4 the metA gene which codes for homoserine O-acetyltransferase,

15.5 the metB gene which codes for cystathionine gamma-synthase,

15.6 the aecD gene which codes for cystathionine gamma-lyase,

15.7 the glyA gene which codes for serine hydroxymethyltransferase

15.8 the pgk gene which codes for 3-phosphoglycerate kinase

15.9 the pyc gene which codes for pyruvate carboxylase

is or are enhanced, in particular over-expressed, are fermented.

16. The process as claimed in claim 15, wherein for the preparation of L-amino acids, in particular L-methionine, coryneform microorganisms which have an additional enhancement of the metY gene by metA are fermented.

17. The process as claimed in claim 8, wherein for the preparation of L-amino acids, in particular L-lysine, coryneform microorganisms which have an additional enhancement of the metY gene by attenuation, in particular reduction in expression, of one or more genes chosen from the group consisting of

17.1 the pck gene which codes for phosphoenol pyruvate carboxykinase

17.2 the pgi gene which codes for glucose 6-phosphate isomerase

17.3 the poxB gene which codes for pyruvate oxidase

are fermented.

18. The process as claimed in claim 9, wherein for the preparation of L-amino acids, in particular L-methionine, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of

18.1 the thrB gene which codes for homoserine kinase

18.2 the ilvA gene which codes for threonine dehydratase

18.3 the thrC gene which codes for threonine synthase

18.4 the ddh gene which codes for meso-diaminopimelate D-dehydrogenase

18.5 the pck gene which codes for phosphoenol pyruvate carboxykinase

18.6 the pgi gene which codes for glucose 6-phosphate isomerase

18.7 the poxB gene which codes for pyruvate oxidase

is or are attenuated or reduced in expression are fermented.

19. Coryneform bacteria in which the metY gene is enhanced, in particular over-expressed.

20. Coryneform bacteria which contain a vector which carries a polynucleotide as claimed in claim 1.

21. The process as claimed in one or more of the preceding claims, wherein microorganisms of the species Corynebacterium glutamicum are employed.

22. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes which code for O-acetylhomoserine sulfhydrylase or have a high similarity with the sequence of the metY gene, wherein the polynucleotide comprising the polynucleotide sequences as claimed in claims 1, 2, 3 or 4 is employed as hybridization probes.

Abstract

The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of

a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and a process for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the metY gene is present in enhanced form, and the use of the polynucleotides which comprise the polynucleotide sequences according to the invention as hybridization probes.

Figure 1: Plasmid pCREmetY

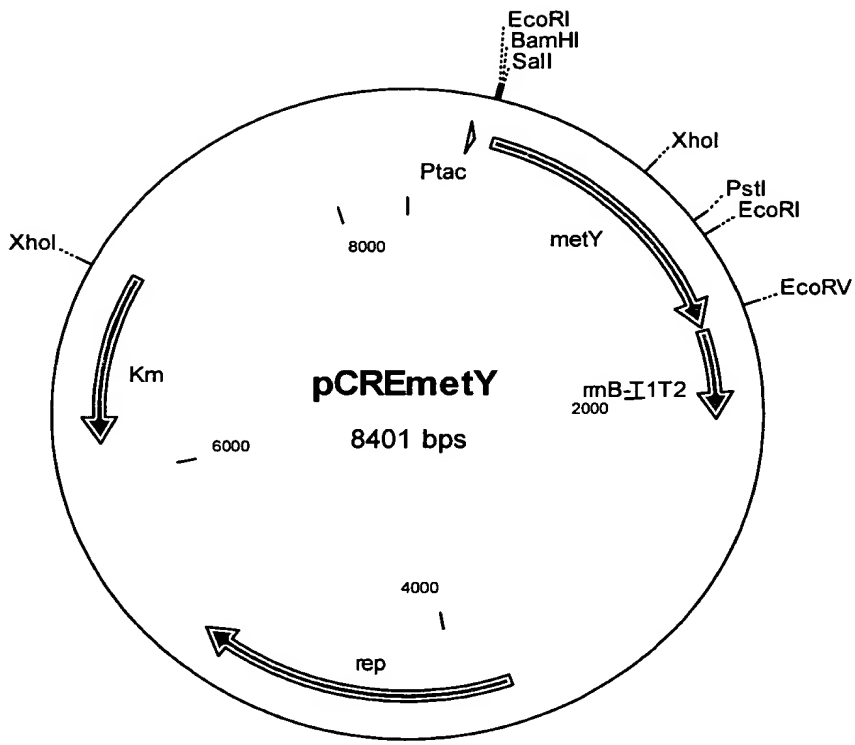
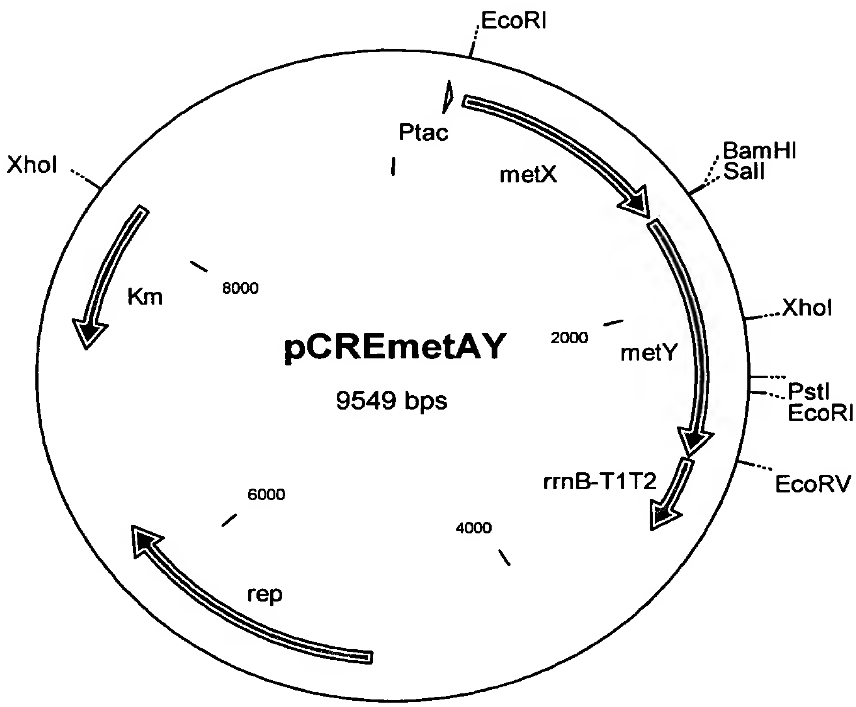


Figure 2: Plasmid pCREmetAY



Translator's notes (000053 BT):

The following errors have been found in the German:

Page 4, lines 18 and 21 - For "Sulfhydrolase" read "Sulfhydrylase"

Page 10, line 26 - For "Oligonukleotide" read "Oligonucleotide"

Page 13, lines 22 and 23 - Repetition of previous two lines

Page 14, lines 19 and 21, page 39, lines 24 and 26 - For "Cystahionin" read "Cystathionin"

Page 16, line 4 - For "Ken" read "Gen"

Page 16, line 15 - For "sind" read "ist"

Claim 20 - For "Sulfhydrolase" read "Sulfhydrylase"